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Effective prevention of lethal acute graft-versus-host disease by combined immunosuppressive therapy with prodigiosin and cyclosporine A

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Abstract

Prodigiosin (PDG), a bacterial metabolite, is a known T cell-specific immunosuppressant. Here, we compared its inhibitory potency and mode of action with cyclosporine A (CsA) in a mouse model. PDG efficiently inhibited T cell proliferation with an IC_{50} of 3.37 ng/ml, a similar dose to that of CsA (IC_{50} of 2.71 ng/ml). PDG inhibited only IL-2Rα expression, but not IL-2 expression, whereas CsA inhibited both. Exogenously added IL-2 reversed the suppressive activity of CsA, but not that of PDG. Moreover, although both PDG and CsA markedly reduced mortality rates in lethal acute graft-versus-host disease (GVHD), the combined treatment was more effective than either drug alone. These results demonstrate that PDG and CsA have similar inhibitory potencies, but different modes of action, and suggest that PDG has potential use as a supplementary immunosuppressant in combination with CsA for the treatment of GVHD.

Keywords: Prodigiosin; Cyclosporin A; Graft-versus-host disease

1. Introduction

Acute graft-versus-host disease (GVHD) is a major cause of morbidity and mortality in patients undergoing allogeneic bone marrow transplantation (BMT) [1]. Acute GVHD begins when a donor's Th1 cells recognize alloantigens presented by host antigen presenting cells (APCs) via interactions of T cell receptor-major histocompatibility, CD28/B7 and CD40/CD40L [2]. The resulting activated Th1 cells secret IL-2 and IFN-γ, which stimulate macrophages and cytotoxic T cells to ultimately produce effector molecules, such as TNF-α, nitric oxide, free radicals, granzyme and perforin, which cause tissue destruction and GVHD. Thus, T cells play a prominent role in the pathogenesis of GVHD.

The interactions between IL-2 and its receptors critically regulate the magnitude and duration of T cell activation [3]. IL-2 exerts multiple biological functions by binding to high-affinity receptors (IL-2R) composed of α, β and common γ chain subunits [4]. Undetectable on resting T cells, α chain expression is triggered by antigen, a stimulus that can be mimicked by Concanavalin A (Con A) or by anti-T cell receptor antibodies. Moreover, IL-2Rα expression is an essential determinant of the acquisition by a cell of full IL-2 responsiveness. Recent intensive studies on the biochemical process of IL-2/IL-2R signal transduction have targeted this pathway for potential pharmacological interventions capable of altering the progression of a broad range of T cell-mediated diseases, including GVHD. Moreover, the blockade of IL-2 transcription by cyclosporin A (CsA) and IL-2-driven signaling by rapamycin have been demonstrated to dampen immunological responsiveness [5,6].

During the past decade, the prodigiosin family has been suggested to be a source of reference compounds for a growing family of drugs with potential therapeutic benefits. Members of this family include prodigiosin (PDG), cycloprodigiosin hydrochloride, undecylprodigiosin and metacycloprodigiosin [7]. They, which contain a

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methoxypyrrole ring, have several biological activities, e.g., as antibacterials, antimycotics, antimalarials and as anticancer agents [7–12]. In addition, many studies imply that they have strong immunosuppressive activity [13–17]. PDG is also known to selectively suppress the immune functions of T cells, but not those of B cells or macrophages. In addition, it has been reported that PDG has a unique mode of action, namely, that it blocks IL-2Rα expression but not IL-2 expression. In contrast, CsA, which is the most well-known T cell-specific immunosuppressant, inhibits IL-2 production by blocking NF-AT activation [18].

Combination immunosuppressive therapies are used to achieve a maximum therapeutic effect whilst minimizing attendant toxicities [15,19]. One prerequisite for the combined use of different drugs is that they should have different modes of action and unrelated toxicities. However, so far no comparative study has been performed on PDG and CsA. Therefore, in the present study, we compared the inhibitory potencies and modes of action of PDG and CsA and further examined the combined therapeutic effects of PDG and CsA on GVHD.

2. Materials and methods

2.1. Materials

Female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from Daehan Biolink Co. Ltd. (Chungbuk, Korea) and maintained under specific pathogen free conditions until required. The experimental procedures used in this study were approved by the KRIBB Animal Experimentation Ethics Committee. PDG (molecular weight 323) was prepared from the culture broth of *Serratia marcescens* [14,20]. Con A and CsA were purchased from Sigma (St. Louis, MO), and mouse recombinant IL-2 was purchased from R & D Systems Inc. (Minneapolis, MN).

2.2. Lymphoproliferation assay

Spleen cells were obtained from specific pathogen free C57BL/6 mice (female, 6–7 weeks) and were freed of red blood cells by lysis buffer treatment. Splenic B cells were isolated by negative depletion by using biotinylated antibodies to CD4, CD8, GR-1 and CD11c (BD Pharmingen) and Dynabeads M-280 Streptoavidin (Dynal Inc., Oslo, Norway), as previously described [21,22]. Splenic T cells were isolated by negative depletion using biotinylated antibodies to B220, GR-1 and CD11c. Purity was typically >90%. Cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine and 50 μM 2-mercaptoethanol (Sigma). T cells were activated with Con A (1 μg/ml) or plate-bound anti-CD3 (10 μg/ml) plus soluble anti-CD28 antibodies (10 μg/ml). B cells were activated with LPS (1 μg/ml) or anti-IgM antibody (10 μg/ml) [23,24]. PDG and CsA were treated at concentrations ranging from 3 to 30 ng/ml. Cells were pulsed with ^{3}H-thymidine (113 Ci/nmol, NEN, Boston, MA) at a concentration of 1 μCi/well for the last 18 h and harvested on day 3 using an automated cell harvester (Inotech, Dottikon, Switzerland). The amount of ^{3}H-thymidine incorporated into cells was measured using a Wallac Microbeta scintillation counter (Wallac, Turku, Finland).

2.3. Cytokine gene expression

Splenic T cells were stimulated with 1 μg/ml of Con A for 24 h and total RNA was extracted using an Ultraspec II RNA isolation Kit (Biotech Lab. Inc., Houston, TX). Reverse transcription-polymerase chain reaction (RT-PCR) was used to examine cytokine gene expressional changes, as described previously [14]. The primer sequences used were as follows: IL-2, sense, 5′-CTT GCC CAA GCA GGC CAC AG-3′, antisense, 5′-GAG CCT TAT GTG TTG TAA GC-3′; IFN-γ, sense, 5′-AGC GGC TGA CTG AAC TCA GAT TGT AG-3′, antisense, 5′-GTC ACA GTT TTC AGC TGT ATA GGG-3′; IL-2Rα, sense, 5′-AAC AAC TGC AAT GAC GGT GA-3′, antisense, 5′-GCC CTC TCT CCC ATT AAA GC-3′; TNF-α, sense, 5′-CCT GTA GCC CAC GTC GTA GC-3′, antisense, 5′-TTG ACC TCA GCG CTG AGT TG-3′; IL-1β, sense, 5′-TGC AGA GTT CCC CAA CTG GTA CAT C-3′, antisense, 5′-GTG CTG CCT AAT GTC CCC TTG AAT C-3′; β-actin, sense, 5′-TGG AAT CCT GTG GCA TCC ATG AAAC-3′, antisense, 5′-TAA AAC GCA GCT CAG TAA CAG TCCG-3′. After analyzing band areas using an image analysis system (Multi-Analyst, Bio-Rad, CA), target mRNA expression levels were calculated as relative ratios versus β-actin [25].

2.4. Lethal acute GVHD

Recipient BALB/c mice received 10 Gy of total body irradiation from a Soft M-150 WE (Softex, Tokyo, Japan) ^{60}Co source at a rate of 0.5 Gy/min [2]. One day after irradiation, the donor mice (C57BL/6 for allogeneic and BALB/c for syngeneic transplantation) were killed by cervical dislocation. Femora were excised aseptically and bone marrow (BM) cells were removed from the femoral shafts by inserting a 25-gauge needle into proximal ends. Total splenic cells were freed of red blood cells by lysis buffer treatment. Irradiated BALB/c mice received a single injection of 0.25 ml of Phosphate Buffered Saline (PBS) containing 1×10^7 BM cells and 5×10^7 spleen cells through a tail vein. BALB/c mice were administered 1 mg/kg of PDG and/or CsA intraperitoneally for 13 days from the day of transplantation.

2.5. Collagen-induced arthritis (CIA)

Male DBA/1 mice were purchased from Charles River Japan Inc. (Yokohama, Japan). Bovine type II collagen was

diluted in 0.05 M acetic acid to 2 mg/ml and emulsified in equal volumes of Complete Freund's Adjuvant (CFA, 2 mg/ml of *Mycobacterium tuberculosis* strain H37RA, Difco, Detroit, ML). The mice ($n = 13$) were initially immunized intradermally at tail base with 100 μl of emulsion. On day 21, the animals were given i.p. booster injections of 100 μg of type II collagen dissolved in PBS. On day 28, arthritis onset was accelerated by a single i.p. injection of 40 μg of LPS. Mice were treated with 10 mg/kg of PDG on alternate days from the day following the LPS injection. Mice were examined visually for the appearance of arthritis in the joints and macroscopic severity scores were given, as previously described [26]. The clinical severity of arthritis was graded on a scale of 0–1.5 for each paw, according to changes in redness and swelling, where 0 = no change, 0.5 = moderate, 1.0 = marked, 1.5 = maximal swelling and redness and ankylosis. The macroscopic score (mean ± S.D.) was expressed as a cumulative value for all paws. On the last day of the experiments, mice were sacrificed by cervical dislocation. Total RNA was extracted from spleen cells using an Ultraspec II RNA isolation Kit (Biotech Lab. Inc.). RT-PCR was used to determine cytokine gene expression changes, as described previously.

2.6. Statistics

The in vivo results represent samples from 6 to 10 mice per experiment, and in vitro results represent the mean values of four samples. All experiments were performed at

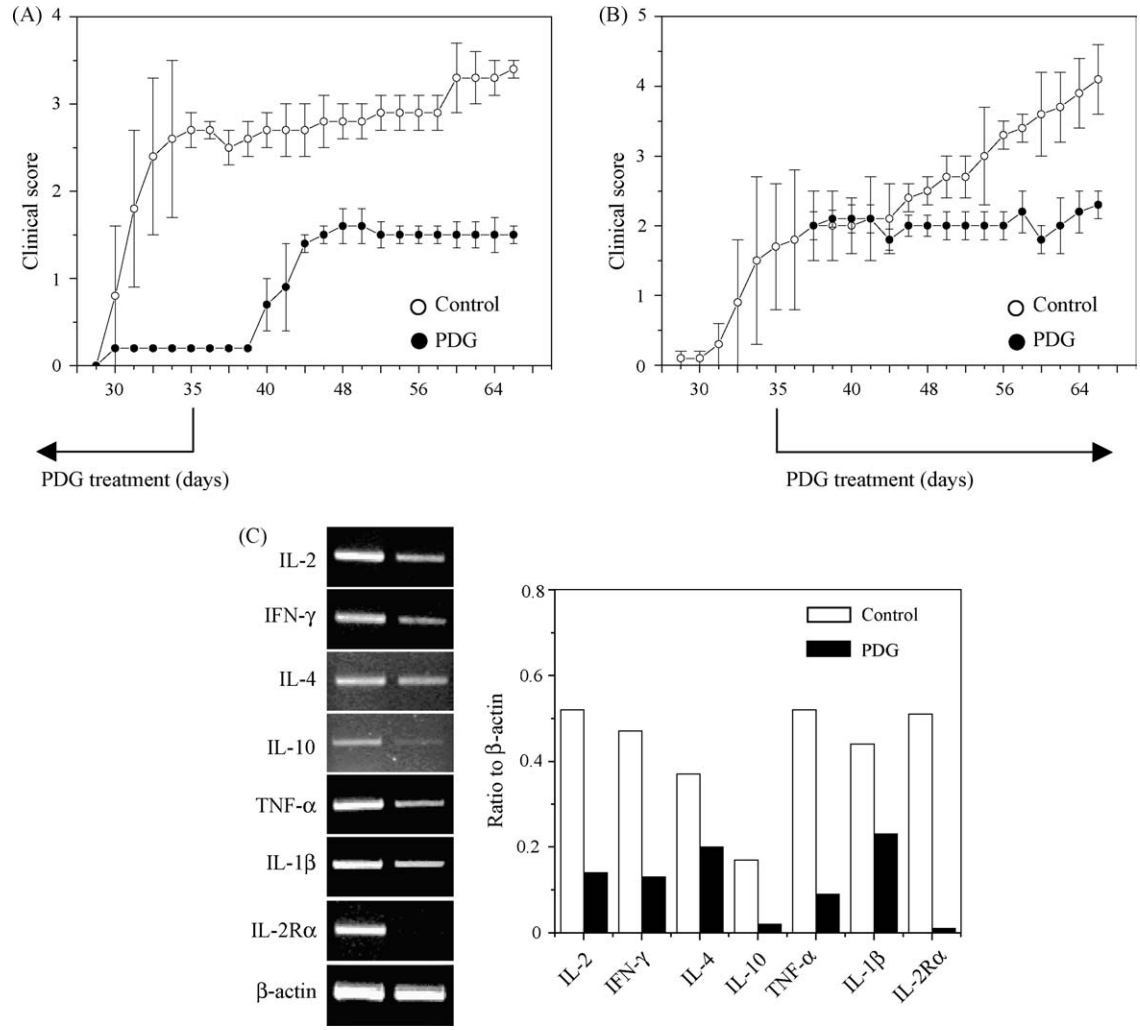

Fig. 1. PDG delays the onset and progression of collagen-induced arthritis. DBA/1 mice were treated with 10 mg/kg of PDG on alternate days from day 29 to day 35 (A) or from day 35 to day 65 (B). Control DBA/1 mice were treated with 0.4% Tween 80, which was used to dissolve PDG. Mice were examined visually for the appearance of arthritis in joints. The clinical severities of arthritis were graded on a scale of 0–1.5 for each paw, according to changes in redness and swelling, where 0 = no change, 0.5 = moderate, 1.0 = marked, 1.5 = maximal swelling and redness, and ankylosis. The macroscopic score (mean ± S.D.) allocated was the cumulative value for all paws. (C) Total RNA was extracted from the spleen cells of DBA/1 mice on day 65 and the gene expression levels of Th1-(IL-2 and IFN-γ), Th2-(IL-4 and IL-10) and of the macrophage-derived cytokines (TNF-α and IL-1β) were analyzed by RT-PCR. IL-2Rα mRNA expression was also determined by RT-PCR. Band areas were analyzed using an image analysis system (Multi-Analyst, Bio-Rad, CA), and data were presented as ratios vs. β-actin, which served as an internal control. "Control" animals were treated with vehicle alone.

least four times. Standard deviations (S.D.s) and p-values were calculated using the Student's t-test in Microsoft Excel®.

3. Results

We previously reported that PDG delayed the progression of CIA [14], and here we investigated in more detail the therapeutic effect of PDG on its onset and progression. DBA/1 mice were immunized with collagen/CFA on day 0 and further boosted with collagen on day 21 and with LPS on day 28. PDG was administered into DBA1 mice in two different ways. First, DBA/1 mice were treated with PDG (at 10 mg/kg) on alternate days from day 29 to day 35, to investigate the effect of PDG on the onset of arthritis. As shown in Fig. 1A, PDG markedly delayed the onset of arthritis, as assessed by macroscopic arthritis scores. To determine whether PDG was effective after the onset of arthritic inflammation, DBA/1 mice were treated with PDG from day 35 to day 65, and this was found to ameliorate arthritis progression (Fig. 1B). In addition to visual scoring, we examined the histological features of joints on the last day of the experiment. PDG was found to have markedly reduced inflammatory cell infiltration (data not shown).

To assess changes in inflammatory cytokine expression levels, total RNA was isolated from the spleen cells of DBA/1 mice used in Fig. 1B. As shown in Fig. 1C, the mRNA expression levels of Th1-(IL-2 and IFN-γ), Th2-(IL-4 and IL-10) and macrophage-derived cytokines (TNF-α and IL-1β) in the spleen cells of PDG-treated mice were markedly reduced versus those in corresponding control spleen cells. In addition, PDG administration reduced IL-2Rα mRNA expression in spleen cells. In summary, these results suggest that PDG delays the onset and the progression of CIA by inhibiting inflammatory cell functions.

To verify that PDG is a generally useful immunosuppressant for the treatment of T cell-involved immunological diseases, we tested its activity on GVHD. In our preliminary experiments, we found that PDG or CsA at high concentrations (>10 mg/kg) completely prevented GVHD (data not shown). However, here we used suboptimal doses of PDG or CsA at 1 mg/kg each in order to investigate the combined therapeutic effect of PDG plus CsA. Lethal acute GVHD was induced by intravenously injecting allogeneic bone marrow and spleen cells from C57BL/6 mice into BALB/c mice. All recipient BALB/c mice without PDG treatment died within 9 days of transplantation (Fig. 2A). In these mice, clinical symptoms of acute GVHD, such as hair ruffling, lowered morbidity and weight loss, became apparent within 2 days (Fig. 2B and data not shown). Moreover, PDG (1 mg/kg) markedly reduced mortality, and 60% of mice survived up to 14 days (Fig. 2A). The protective effect of PDG on weight loss was not apparent until day 6, but body weights

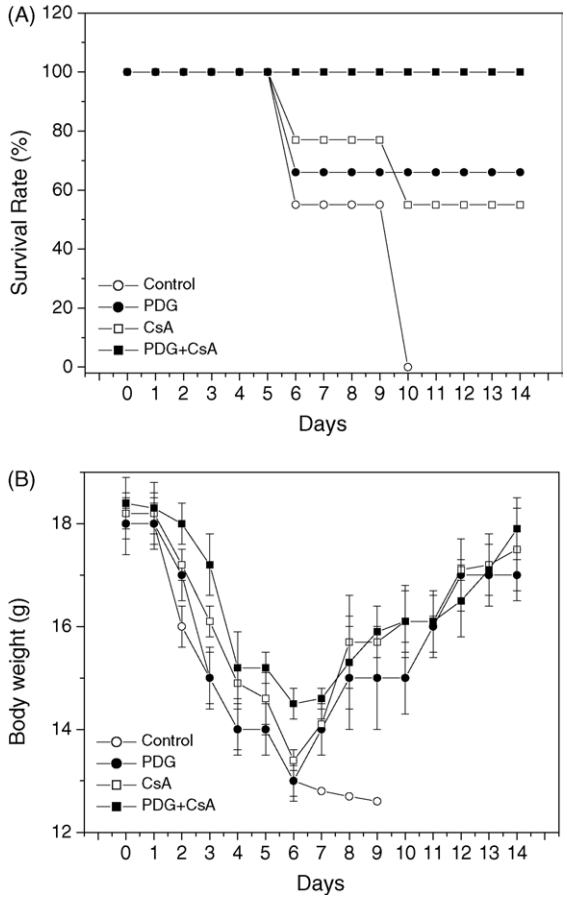

Fig. 2. PDG alone or in combination with CsA prevents acute lethal GVHD. GVHD was induced by intravenously injecting bone marrow and spleen cells from C57BL/6 mice into BALB/c mice ($n = 6$) administered 10 Gy of total body irradiation, as described in Section 2. BALB/c mice were treated on alternate days with PDG (1 mg/kg), CsA (1 mg/kg) (open circles) or PDG plus CsA (both 1 mg/kg) for 13 days. Control BALB/c mice were treated with 0.4% Tween 80, which was used to dissolve the PDG and CsA. The survival rates (A) and body weights of the live mice (B) were recorded daily.

began to rise at day 7 and reached normal levels at day 12 (Fig. 2B). The administration of CsA (1 mg/kg) reduced mortality rate in the same manner as PDG (Fig. 2A). However, the PDG (1 mg/kg) plus CsA (1 mg/kg) produced much better effects than either drug alone, and all mice survived for up to 14 days (Fig. 2A). All syngeneic transplant mice also survived for more than 14 days; these mice also suffered weight loss in the same manner as PDG-treated mice (data not shown). In summary, these results suggest that PDG can prevent GVHD and CIA (both T cell-related diseases), and that PDG plus CsA prevents GVHD more effectively than either drug alone.

We then compared the modes of action of these two agents, with a focus on the IL-2/IL-2R signaling pathway, which critically regulates T cell activation, and also compared inhibitory potencies by using a lymphoproliferation assay. The concentrations of PDG or CsA used ranged from 3 to 30 ng/ml, as this was determined to be a non-toxic range in our previous study [14]. As shown in

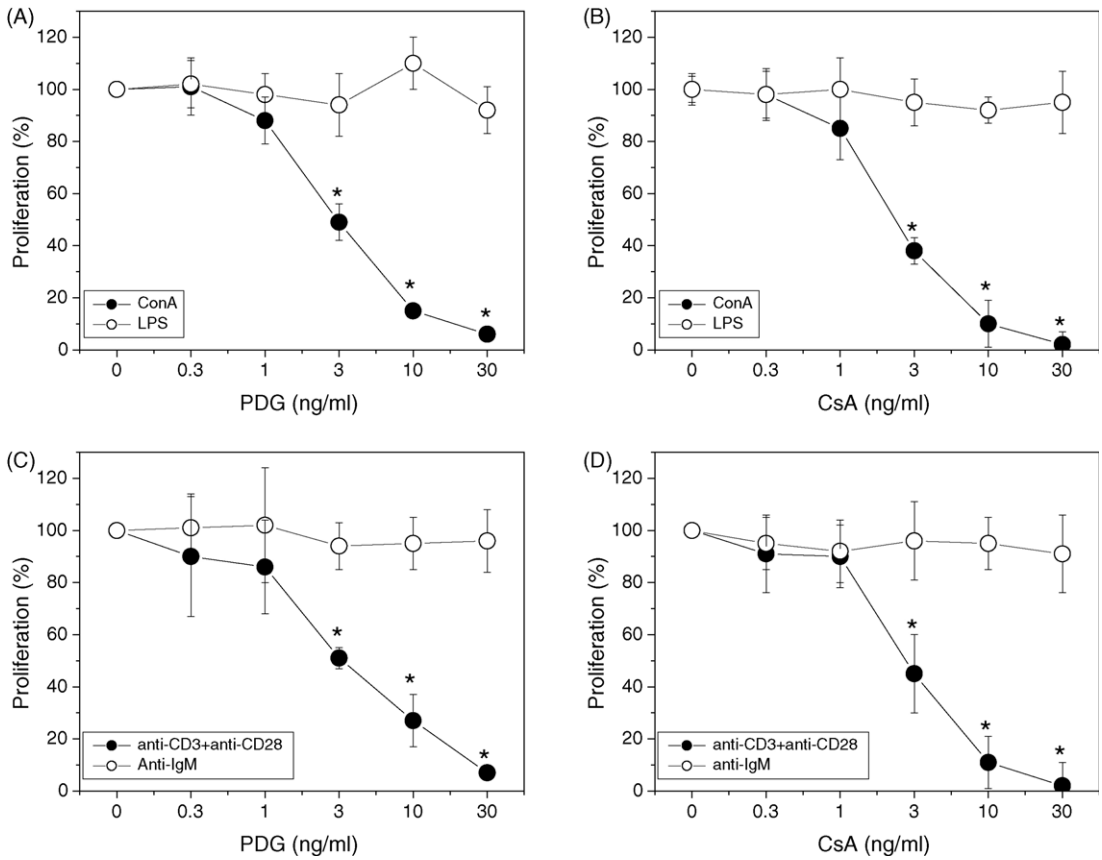

Fig. 3. PDG and CsA selectively inhibit T cell proliferation. Purified T cells were activated with Con A (1 μg/ml) or plate-bound anti-CD3 (10 μg/ml) plus soluble anti-CD28 antibodies (10 μg/ml). Purified B cells were activated with LPS (1 μg/ml) or anti-IgM antibody (10 μg/ml). PDG (A and C) and CsA (B and D) were dissolved in dimethyl sulfoxide (DMSO) and added simultaneously with ConA. Degrees of lymphocyte proliferation were measured by incorporating ^{3}H-thymidine into cellular DNA. The count per minute (CPM) values of vehicle-treated controls were 43,325 ± 437 for Con A, 41,458 ± 981 for LPS, 93,317 ± 943 for anti-CD3 plus anti-CD28 and 63,365 ± 1246 for anti-IgM. Significance was determined using the Student's *t*-test vs. the vehicle (0.1% DMSO)-treated controls ($^{*}p < 0.01$).

Fig. 3A and C, PDG strongly inhibited T cell proliferation, whereas B cell proliferation was unaffected by PDG. CsA inhibited T cell proliferation in a similar manner to PDG (Fig. 3B and D). PDG had a mean IC_{50} of 3.37 ng/ml and CsA a mean IC_{50} of 2.71 ng/ml in T cells.

In the following experiments, we directly compared the effects of PDG and CsA on IL-2 mRNA expression in T cells. PDG did not block Con A-induced IL-2 gene (Fig. 4A) or protein expression (data not shown), whereas CsA completely inhibited their expressions. We also compared the effects of PDG and CsA on IL-2Rα mRNA in T cells, and found that IL-2Rα mRNA expression was strongly inhibited by both PDG and CsA, as determined by RT-PCR (Fig. 4B). Summarizing, these results suggest that PDG and CsA have different modes of action, as PDG inhibited IL-2Rα expression, but not that of IL-2, whereas CsA blocked both.

The above results suggest that the suppressive activities of PDG and CsA are independent and dependent on IL-2, respectively. This result was further confirmed by determining the effects of exogenously added IL-2 (10 unit/ml) on the immunosuppressive activities of PDG or CsA. PDG activity on T cell proliferation was unchanged in the presence or absence of exogenous IL-2 (Fig. 5A), whereas CsA activity was recovered by adding exogenous IL-2 (Fig. 5B). We then investigated the effect of PDG on IL-2-induced T cell proliferation. Accordingly, T cells were preincubated with Con A to make them responsive to IL-2. However, neither PDG nor CsA inhibited IL-2-induced T cell proliferation (Fig. 5C and D). In summary the above data suggest that PDG may inhibit unidentified signaling molecules specifically targeting IL-2Rα expression, and may not inhibit calcineurin or NF-AT, which are specific targets of CsA.

4. Discussion

The present study demonstrates that PDG and CsA have similar inhibitory potencies on T cell proliferation with IC_{50}'s of 3.37 ng/ml and 2.71 ng/ml, respectively. However, the study also shows that they have different modes of action. PDG inhibits only IL-2Rα expression, whereas CsA inhibits both IL-2Rα and IL-2. Moreover, exogenously added IL-2 reversed the suppressive activity of CsA, but not that of PDG. Finally, our results demonstrate the

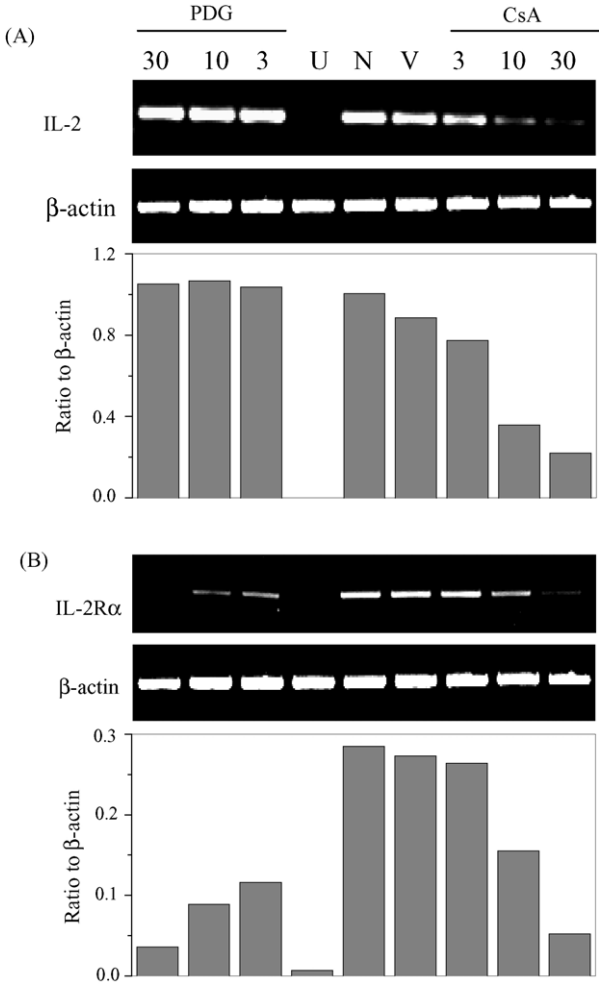

Fig. 4. PDG inhibits IL-2Rα expression, but not IL-2 expression. Splenic T cells were activated with 1 μg/ml of Con A for 4 h. PDG and CsA were dissolved in DMSO and added simultaneously with ConA. Total RNA was extracted and the gene expression levels of IL-2 (A) and IL-2Rα (B) were analyzed by RT-PCR. U, chemically-untreated control cells; N, Con A-treated naive control cells; V, vehicle (0.1% DMSO)-treated controls.

therapeutic effect of PDG alone and in combination with CsA for the prevention of GVHD.

Recently, prodigiosin analogues have attracted considerable attention because of their promising physiological properties, particularly their anticancer and immunosuppressive activities [27]. They have been found to induce the apoptosis of several cancer cells [9–11], and the National Cancer Institute (USA) obtained an average IC_{50} of 2.1 μM for PDG against a panel of 57 different human cancer cells [28]. However, prodigiosin analogues usually inhibit T cell functions at much lower doses than are required for cancer cell apoptosis. The PDG used in this study showed strong immunosuppressive effect at <30 ng/ml, which is obviously non-toxic to lymphocytes [14]. Regarding the adverse effects of PDG in vivo, we found that PDG did not induce any observable adverse effects at biologically effective doses. For example, it did not induce body weight losses and any other gross change in mice administered PDG i.p. for 30 days. However, further investigations on organ-specific toxicity should be undertaken to determine therapeutic indexes at maximum tolerance doses versus pharmacologically effective doses. Our preliminary experiments showed that single i.p. injection of PDG to ICR mice showed a median lethal dose (LD_{50}) of 149 mg/kg. PDG at 100 mg/kg showed no weight changes in the liver and kidney, but did show some necrotic cells in the liver. In daily i.p. injection of 100 mg/kg of PDG for 1 week, we found no weight changes in the liver and kidney, but did find spleen weight loss and some necrotic cell death in the liver. However, there were no appreciable changes of weight and gross clinical signs in the liver and kidney at biologically effective doses from 1 to 30 mg/kg of PDG. Nevertheless, we still could not exclude the possible liver/kidney damage after 1-month treatment.

Our data extend previous observations concerning the delay of CIA progression by PDG [14]. In the present study, we examined the effect of PDG on CIA in more detail and found that it delays both the onset and progression of arthritic disease. As a possible mechanism, we demonstrate that PDG in vivo can inhibit inflammatory cytokine production by T cells and macrophages, which are critical for the development of CIA. The mRNA expression levels of Th1-(IL-2 and IFN-γ), Th2-(IL-4 and IL-10) and macrophage-derived cytokines (TNF-α and IL-1β) in spleen cells from PDG-treated mice were markedly reduced versus corresponding controls. In addition, the administration of PDG reduced IL-2Rα mRNA expression in spleen cells. In our previous study, we showed that PDG in vitro did not directly inhibit IL-2 production by Th1 cells, or TNF-α and IL-1β production by macrophages [14]. Our preliminary data also showed that PDG did not inhibit macrophage TNF-α production in LPS-injected mice, when we administered PDG 30 min before LPS injection (data not shown). A possible explanation for this discrepancy between previous in vitro and the present in vivo data is that prolonged PDG treatment may induce sequential inhibition of IL-2Rα expression in T cells, cytokine production in T cells and cytokine production in macrophages. It may be related with the fact that IL-2Rα expression is an essential determinant in T cell activation and T cell-derived cytokines play a key role in macrophage activation. However, we could not exclude the possibility that PDG might show toxicity and related immunosuppression in macrophages after prolonged PDG administration.

Several therapeutic approaches have been designed by targeting T cell activation to reduce acute GVHD. The removal of donor T cells from marrow grafts has been reported to reduce GVHD [29]. Attempts to block the interaction between T cells and APCs also appeared to be highly effective at preventing acute GVHD. Moreover, the administration of antibodies against CD80, CD86 and/or CTLA4Ig (CD28) was found to effectively reduce the

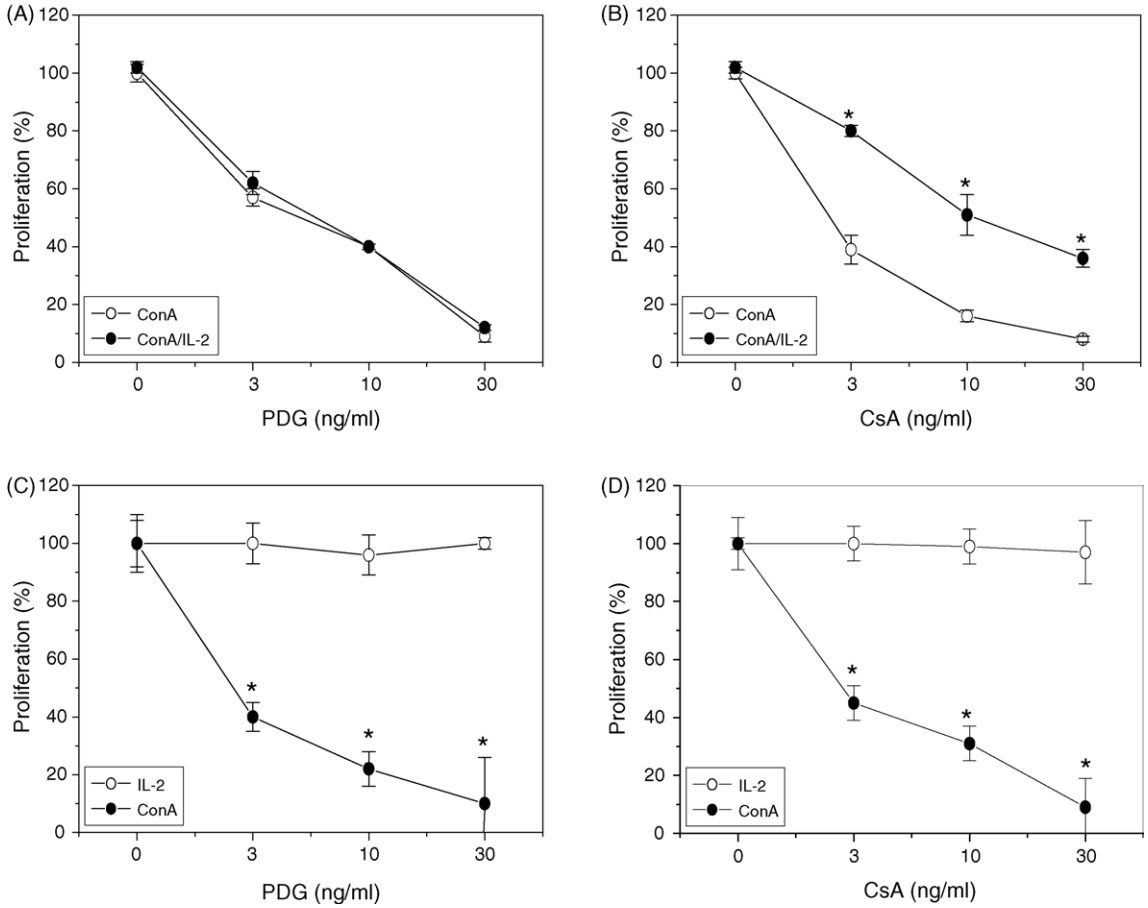

Fig. 5. Exogenous IL-2 reverses the suppressive activity of CsA, but not that of PDG. (A and B) Splenic T cells were activated with Con A (1 μg/ml) alone or in combination with IL-2 (10 unit/ml) for 72 h. PDG (A) and CsA (B) were dissolved in DMSO and added simultaneously with stimuli (ConA or ConA plus IL-2) from the beginning of culture. Degrees of lymphocyte proliferation were measured by incorporating ^{3}H-thymidine into the cellular DNA. The count per minute (CPM) values of control groups were 33,705 ± 760 (Con A) and 34,010 ± 945 (Con A/IL-2). Significance of the results of Con A/IL-2-treated groups was determined using the Student's *t*-test vs. the Con A-treated group ($^{*}p < 0.01$). (C and D) Splenic T cells were preincubated with Con A (1 μg/ml) for 48 h. After complete washing, these cells were rested for 24 h and further activated with IL-2 (10 units/ml) for 72 h. PDG (C) and CsA (D) were added simultaneously with the IL-2. As control experiments, fresh splenic T cells were activated with 1 μg/ml Con A. PDG (C) and CsA (D) were added simultaneously with ConA. Degrees of lymphocyte proliferation were measured by incorporating ^{3}H-thymidine into cellular DNA. The CPM values of the control were 47,618 ± 1421 (IL-2) and 37,112 ± 1045 (Con A). Significance of the results of the Con A/IL-2-treated group was determined using the Student's *t*-test vs. the vehicle-treated control group ($^{*}p < 0.01$).

mortality rate due to GVHD [30,31]. Similarly, antibodies to IL-2 and IFN-γ have also been reported to prevent GVHD in mice [32,33]. An alternative approach to preventing acute GVHD without using antibodies involves the administration of T cell-specific immunosuppressants [19,34]. CsA and rapamycin, which are both chemical immunosuppressants that inhibit the IL-2/IL-2R signaling cascades in T cells, have been shown to effectively reduce GVHD lethality in murine models. However, none of the therapeutic strategies has resulted in the complete prevention of acute GVHD. The administration of antibodies to block CD28/B7 interaction or cytokine (IL-2 or IFN-γ) expression appears to reduce mortality rates by 50–70% [32,33,35]. This seems to be because not all T cells are dependent upon CD28/B7 costimulation and because each antibody neutralizes only one cytokine. The suppression of T cell activation by administering high doses of CsA may result in the complete inhibition of acute lethal GVHD, but only at the cost of inducing diverse adverse effects [36].

In order to overcome the fundamental problem of intrinsic toxicity, shared by almost all chemical immunosuppressants, two or more immunosuppressive drugs were used in combination to permit the use of relatively low nontoxic drug concentrations [15,19]. It is a prerequisite of combinatorial regimens that the different drugs should have different receptors, intracellular targets and modes of action [37]. CsA dominates current combination immunosuppressive therapies, and can specifically disrupt the phosphatase activity of calcineurin dephosphorylating nuclear factor of activated-T cell (NF-AT), which is critical for IL-2 gene expression [38]. However, prodigiosin has not been found to affect NF-AT activation [14]. Although the molecular targets of prodigiosin analogues

have not yet been clearly identified, the inhibitions of JAK3, retinoblastoma protein phosphorylation, NF-kappaB and AP-1 have been implicated in immunosuppression by prodigiosin analogues [17,37,39]. We also suggest that one of the signaling molecules for IL-2Rα expression may be a molecular target of PDG. However, molecular targets of PDG may be not related with IL-2-deriven signalings, since it does not inhibit IL-2-deriven T cell proliferation. Overall, these results suggest that PDG and CsA have different intracellular targets and modes of action, which strongly suggests that PDG could be used as a supplementary immunosuppressant in combination with CsA. In fact, we provide direct evidence of the combination effects of PDG and CsA, and demonstrate that the combined administration of PDG and CsA at low dosages effectively prevented mortality in GVHD.

We report here that PDG inhibits T cell activation by blocking the IL-2/IL-2R signaling pathway in a different manner from, but in a similar potency with CsA, and that combined PDG and CsA treatment might effectively prevent T-cell related immune diseases, including GVHD. Further studies will focus on the identification of ultimate molecular targets of PDG in T cells.

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